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PHOSPHOPROTEIN METABOLISM IN CEREBRAL FUNCTIONING

from

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SPECIAL TECHNICAL REPORT. I.

The research reported in this document has been made possible through the support and sponsorship of the U.S. Department of Army, through its European Research Office, Contract No.: DA-91-591-EUC-1944.

Abstract

The report describes investigations into the following three aspects of cerebral phosphoproteins and their metabolism.

The subcellular distribution of cerebral phosphoproteins

In earlier work it was shown that electrical stimulation of slices of guinea pig cerebral cortex, respiring with inorganic ^{32}phosphate, results in an increase in the radioactivity of the tissue phosphoprotein-P. Experiments designed to determine the subcellular site of the phosphoprotein sensitive to stimulation suggested it was located in structures within the nuclear fraction, but interpretation proved difficult, since the nuclear fraction was contaminated with material from other fractions.

As a preliminary to the present investigation, the subcellular distribution of phosphoproteins in brain was determined by procedures yielding well-defined fractions. Fresh cerebral cortex homogenates were fractionated by differential centrifuging into 5 primary fractions. In each fraction the ratio mμmoles phosphoprotein-P to mg. protein was determined. Mean values for 7 experiments were as follows: nuclear, 10.7 ± 0.3; mitochondrial I, 6.0 ± 1.8; mitochondrial II, 7.2 ± 1.0; microsomal, 12.3 ± 2.2; supernatant, 10.1 ± 1.4

The nature of the nuclear subfraction believed to contain the sensitive phosphoprotein was then explored. Primary nuclear and mitochondrial fractions were subfractionated by density-gradient methods. Four subfractions (A B, C, and D) were isolated from both primary fractions and analysed for protein, phosphoprotein-P, RNA, cholesterol, and succinic dehydrogenase. The analyses suggested that nuclear subfractions A, B, and C largely consisted of cross-contaminating material from the mitochondrial fraction. Thus nuclear subfraction B, considered from the earlier work to be rich in the sensitive phosphoprotein, corresponded in composition to mitochondrial subfraction B. By washing the primary nuclear fraction 3 times by resuspension and recentrifuging, at least 90% of A, B, and C were removed without appreciably affecting D. Electron microscopy of the subfractions also tended to confirm this conclusion. Shrunken nerve-endings and damaged mitochondria were seen in both B-subfractions, but the nuclear subfraction appeared more heterogeneous.

The subcellular distribution of phosphoprotein-^{32}P following electrical stimulation of cortex slices was studied by determining the radioactivity of phosphorylserine isolated from protein residues by acid hydrolysis and chromatography. Slices were electrically stimulated in quick-transfer holders under conditions established in an earlier study.

In preliminary experiments, homogenates of tissue were centrifuged in a sucrose density gradient. In three experiments the specific activity of phosphoprotein-P in a subfraction equilibrating above 1.2 M-sucrose (B) was found to increase by an average of 73% on stimulation, compared with an increase of 48% in the fraction equilibrating above 1.4 M-sucrose (C). Since the average succinic dehydrogenase activity in subfraction C was twice that of subfraction B, it appears unlikely that a major increase in the radioactivity of mitochondrial phosphoprotein occurs on stimulation. The distribution of phosphoprotein radioactivity was further examined by differential centrifugation. It was observed that the distribution of particulate material obtained from incubated tissue was affected by small quantities of medium carried to the sucrose by the slice, the nuclear fraction increasing in protein content at the expense of the mitochondrial and microsomal fractions. When slices were rinsed in 0.32 M-sucrose, a procedure subsequently adopted, the yield of microsomal protein increased from

0.2 mg./gm. of tissue to 10 - 12 mg./gm. Homogenates of incubated tissue were centrifuged to obtain a combined nuclear and mitochondrial fraction (F1; 2×10^5 g min.) and a microsomal fraction (F2; 6.2×10^6 g min.). In 6 experiments, the increase in phosphoprotein radioactivity following stimulation averaged 30 ± 3.5% in the homogenate, 5.2 ± 3.2 in F1, and 23.7 ± 5.5 in F2. These results suggest that a sensitive phosphoprotein is located in membrane structures normally sedimented with a microsomal fraction rather than a nuclear fraction as earlier claimed.

Cerebral protein phosphokinase

The properties of a protein phosphokinase enzyme from ox brain, catalysing the transfer of inorganic phosphate from ATP to phosvitin, were examined. The enzyme was prepared from acetone powders of ox brain grey matter and effect of cations on activity assayed by measuring ^{32}P transferred in 10 min. to 2 mg. of phosvitin from 1 µmole of $AT^{32}P$ buffered with 50 mM-tris-HCl, pH 7.4. The enzyme was stimulated by Mg, maximally at 5 mM-; higher concentrations were progressively inhibitory. With 5 mM-Mg present, a further stimulation, ranging from 150 - 220% of control was given by the following ions (at 200 mM-) in order of effectiveness: K, Rb, Cs, Na, NH_4, choline, and tris. Under the same conditions, Li was inactive and Ca (1 - 5 mM-) inhibitory. The percentage stimulations shown by K, Rb, Cs, and Na increased as the Mg concentration was lowered from 5 to 1 mM-. Both Na and K gave maximal stimulation at 200 mM- in the range 1 - 5 mM-Mg.

The subcellular distribution of the enzyme was studied, using the same basic system. Of the total activity in homogenates of guinea pig cerebral cortex, approximately 46% was found in the soluble fraction (S), 20% in a microsomal fraction (Ms, 9×10^6 g min.), 28% in a mitochondrial fraction (Mt, 2×10^5 g min.), and 6% in a nuclear fraction (N, 8×10^3 g min.). In terms of µmoles P transferred/mg. protein, the activities for the four fractions were: N, 0.049; Mt, 0.028; Mc, 0.058; S, 0.12. Enzyme activity in microsomal and soluble proteins was stimulated by Na and K to approximately the same extent.

Attempts to prepare protein fractions enriched in phosphoproteins

A method was developed for solubilizing between 10 and 20% of the proteins and phosphoproteins from a cerebral membrane fraction and from microsomes. This involved raising the pH of an aqueous suspension of the insoluble lipoproteins to 10.5 at 0° with dilute NaOH, immediately neutralizing and centrifuging at high speed. In some experiments the soluble fraction was reprecipitated at pH 4.5 and partially brought back into solution at pH 7. The resulting soluble fraction was relatively low in phospholipid and slightly enriched in phosphoprotein.

Contents

General Introduction

The studies described in this Report originate from an observation made in this Department by P. J. Heald (1957). Heald found that electrical stimulation for 10 sec. of slices of guinea pig cerebral cortex, respiring *in vitro* in the presence of O_2, glucose, and ^{32}P, results in an increase in the specific radioactivity of the tissue phosphoprotein-P without any change in its concentration. This change occurs concomitantly with a decrease in both the specific radioactivity and concentration of adenosinetriphosphate (ATP) and creatine phosphate in the tissue. The changes in phosphoprotein radioactivity may reflect a role for phosphoproteins in energy-yielding processes concerned in the resynthesis of ATP (Wadkins & Lehninger, 1958) or alternatively an involvement in some aspect of energy utilization in the cell. So far as the latter is concerned, the most likely process would be that of ion transport, since electrical stimulation under these conditions induces changes in ionic balance consisting in a loss of potassium and a gain of sodium by the tissue (Cummins & McIlwain, 1961), and restoration of this balance by the active extrusion of sodium from the cell is probably the major energy-consuming reaction in cerebral tissue (Whittam, 1962). The hypothesis of a role for phosphoproteins in sodium transport is an attractive one in view of the very considerable evidence that a membrane adenosinetriphosphatase (ATPase) plays a part in this process (Skou, 1961). It is noteworthy that phosphorylated intermediates have been postulated to explain ATPase action in mitochondria (Lehninger, 1962), and for muscle myosin (Levy & Koshland, 1959).

The present investigation is divided into three parts, as follows. First, a study of the subcellular location of phosphoproteins in cerebral tissue, especially of the phosphoproteins sensitive to electrical stimulation; second, observations on the properties of enzymes in cerebral tissue transferring P from ATP to phosphoproteins; and finally some preliminary attempts to prepare from cerebral tissue protein fractions enriched in phosphoproteins, with a view to the eventual isolation of the phosphoprotein sensitive to stimulation. In the Report, the three parts are treated separately so far as introductory matter and presentation are concerned. For convenience, however, all experimental methods are included in the next section, and the results are discussed together in a later section.

Experimental materials and methods

Materials

Phosvitin. The product supplied by Nutritional Biochemical Corporation was treated with EDTA and dialysed by the procedure of Rose & Heald (1961).

AT^{32}P. This was prepared essentially by the procedure of Pressman (1960), using disodium ATP from Sigma Chemical Co. The final product was converted to the tris-salt with Dowex 50 resin. Before use the product was diluted with 10 or 20 mM-tris-ATP to the desired concentration and activity.

GT^{32}P. The procedure was esentially the same, except that a mixture of ATP and GTP was present in the reaction mixture. The labelled nucleotides were separated on Dowex 1 x 8 resin and the purity of the GTP fraction checked by rechromatography.

General methods

Dispersions of tissues, acetone powders or protein fractions were made in glass homogenizers fitted with glass or Teflon pestles (A. H. Thomas, Springfield, U.S.).

Centrifugation at high speed was carried out in the superspeed head of a Measuring & Scientific Equipment (MSE) refrigerated centrifuge, in a Spinco Model 'L' Centrifuge or, in later experiments, in a MSE '25000' machine. Centrifugal forces are expressed throughout as g-min., i.e. average g multiplied by the time of centrifugation in min.

Analytical methods

Phosphorous. The isobutanol-benzene method of Martin & Doty (1949) was adapted to suit the scale of the particular experiment. This was usually in the range of 1-10 μg. of P.

Phosphoprotein. This was determined by measurement of the alkali-labile-P in the insoluble residue remaining after extraction of the tissue with 10% (w/v) trichloroacetic acid and 2:1 (v/v) chloroform-methanol (McIlwain & Rodnight, 1962).

Phosphoprotein radioactivity. The above procedure is unsatisfactory for measurement of the specific radioactivity of the protein-P, since the acid-insoluble residue tends to adsorb ^{32}P non-specifically. Provided that an absolute value for phosphoprotein-P is not required, analysis of the phosphoryl serine released by partial acid hydrolysis of the proteins overcomes this difficulty. The procedure used was similar

to that of Heald (1958), except that in every case the actual specific activity of the phosphoryl serine-P was determined, rather than the total radioactivity in the phosphoryl serine fraction. Hydrolysis at 100° was in 2 N-HCl for 5 hr. in sealed tubes. Control experiments showed negligible contamination of the final fraction from the resin column with ^{32}P, but the actual amount of phosphoryl serine obtained in the acid hydrolysates was somewhat variable.

Protein. This was either determined by a Biuret procedure (see Aldridge & Johnson, 1959), or on a micro scale by the method of Lowry et al. (1951). The latter was usually employed in the enzyme studies.

Lipid-P. The procedure given by McIlwain & Rodnight (1962) was used.

Ribonucleic acid (RNA). An approximate measure of RNA was obtained by measuring the absorption at 260 mμ of an alkaline-digest of the acid-insoluble tissue residue, neutralized with $HClO_4$. Yeast-RNA was used as a standard.

Cholesterol. The digitonin method described by McIlwain & Rodnight (1962) was used.

Succinic dehydrogenase. This was determined manometrically using ferricyanide as electron acceptor (Aldridge & Johnson, 1959). To ensure that maximum activity was released from the tissue, samples were frozen and thawed several times and then diluted with water before analysis.

Subcellular fractionations. Dispersions of tissue were made in 10 vol. (w/v) of 0.32 M-sucrose at 0° containing 0.5 mM-EDTA adjusted to pH 7. The dispersion was then fractionated by the various procedures indicated in the text.

Subcellular location of phosphoproteins

Introduction

It was hoped that these studies would indicate whether the increased phosphoprotein metabolism was part of an energy-yielding or an energy-utilizing process. Thus a mitochondrial location would suggest a role in the synthesis of ATP through oxidative phosphorylation, whilst a location in membrane structures would favour a role in ion transport, an energy-consuming process.

Some preliminary studies of these aspects had already been made by Heald (1959, 1961) when the work commenced. Heald showed clearly that the major increase in phosphoprotein radioactivity on electrical stimulation occurred in the proteins of particulate matter rather than in the soluble cytoplasmic proteins.* From indirect evidence, not embracing stimulation, Heald further concluded that the sensitive phosphoprotein occurs in a sub-fraction of the nuclear fraction, distinct from nuclei, mitochondria or microsomes. Interpretation of Heald's results has proved difficult, however, since the crude nuclear fraction he used was apparently grossly contaminated with material from other fractions. Before proceeding to study the composition of the nuclear sub-fraction described by Heald therefore, it seemed desirable to re-investigate the general subcellular distribution of phosphoproteins in brain by procedures yielding well-defined fractions.

In later experiments, the location of the sensitive phosphoprotein was studied directly by stimulating the tissue electrically in the presence of ^{32}P and then sub-fractionating by modified procedures.

Subcellular distribution of phosphoproteins

Fresh guinea pig cerebral cortex was homogenized in 0.32 M-sucrose containing 0.5 mM-EDTA at pH 7 and fractionated by differential centrifuging into 5 primary fractions. The nuclear fraction was washed three times by re-suspension and centrifuging, and the two mitochondrial fractions were similarly each washed twice. In each fraction the ratio mμmoles of phosphoprotein-P (as alkali-labile-P)/mg. of protein was determined and the mean values for 7 experiments are given below.

* For additional comment on this point, see page 8.

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* For additional comment on this point, see page 8.

Table 1. Subcellular distribution of cerebral phosphoproteins

Fraction isolated at centrifugal speed stated	mμmoles of phosphoprotein-P/mg. of protein ± standard deviation
Homogenate	9.7 ± 0.2
Nuclear (8×10^3 g min.)	10.7 ± 0.3
Mitochondrial I (10^5 g min.)	6.0 ± 1.8
Mitochondrial II (2×10^5 g min.)	7.2 ± 1.0
Microsomal (7×10^6 g min.)	12.3 ± 2.2
Supernatant (soluble)	10.1 ± 1.4

These results show clearly that in terms of protein, the microsomes are richest in phosphoproteins. It should be emphasised, however, that the microsomal phosphoproteins only constitute some 12% of the total in the tissue. Corresponding figures for the nuclear fraction are 15%, the mitochondrial fractions (I and II) 30%, and the supernatant (soluble) fraction 30%.

Studies on the nature of the nuclear subfraction

The conclusions reached by Heald (1959, 1961) were based on analysis of a nuclear fraction isolated from dispersions of either fresh or incubated cerebral cortex at 15×10^3 g min, i.e. at approx. twice the force usually used (see McIlwain & Rodnight,1962). In these experiments, however, where direct comparison with Heald's results was important, we used the higher centrifugal force. As before, fresh guinea pig cerebral cortex was used. The crude nuclear fraction was washed once and then sub-fractionated in a sucrose density gradient as described by Heald (1959); the preliminary layering of the fraction over M-sucrose was found unnecessary and was omitted. This procedure of sub-fractionating the nuclear fraction is almost identical with that employed by Whittaker (1959) to sub-fractionate the mitochondrial fraction from cerebral tissue into fractions containing principally mitochondria and fractions rich in nerve endings and acetylcholine. It appeared possible that certain of the sub-fractions from the two crude fractions, equilibrating in the same concentration of sucrose, might prove to be similar in composition. A crude mitochondrial fraction was, therefore, isolated from the dispersions and submitted in parallel to exactly the same sub-fractionation procedure. From each crude fraction, 4 sub-fractions were obtained and these were analysed for phosphoprotein-P, RNA, cholesterol, and SDH. The results are given in Tables 2 and 3 below. When standard deviations are given, the means are derived from five distinct experiments. In all other cases, the results are means of three determinations in distinct experiments.

Table 2. Distribution of phosphoprotein in subcellular fractions of guinea pig cerebral cortex

Parent fraction isolated at g min. stated	Subfraction not sedimenting at 7.5×10^6 g min. through sucrose molarity stated	Protein, mg. derived from 1 g. of cortex	Phosphoprotein mμmoles/mg. protein
Nuclear (15×10^3)	A, 1.0	0.9	14.9
	B, 1.2	2.6 ± 1.1	9.5 ± 1.1
	C, 1.4	2.6 ± 0.58	7.2 ± 2.1
	D, > 1.4	2.3	9.2
Mitochondrial (2×10^5)	A, 1.0	14.6	9.3
	B, 1.2	13.3	9.7
	C, 1.4	9.2	6.6
	D, > 1.4	3.2	7.6

Table 3. Distribution of RNA, cholesterol, and succinic dehydrogenase in subcellular fractions of guinea pig cerebral cortex

Parent fraction isolated at g min. stated	Subfraction not sedimenting at 7.5×10^6 g min. through sucrose molarity stated	RNA μg./mg. protein	Cholesterol μg/mg. protein	SDH μl. CO_2/mg. protein/hr.
Nuclear (15×10^3)	A, 1.0	26.2	224	0
	B, 1.2	12.1 ±3.3	118 ± 20.5	123
	C, 1.4	13.3 ±3.3	85 ± 27.4	232
	D, > 1.4	19.6	80	75
Mitochondrial (2×10^5)	A, 1.0	13.1	158	15
	B, 1.2	17.9	108	148
	C, 1.4	22.8	77	279
	D, > 1.4	17.0	52	0

The nuclear subfraction considered by Heald to contain the sensitive phosphoprotein (see Heald, 1961) corresponds in these fractions to subfraction B, equilibrating above 1.2 M-sucrose. In terms of protein, this contains more phosphoprotein and cholesterol and less SDH than subfraction C. The same relationship between subfractions B and C is seen in the mitochondrial fraction. The figures for RNA show a similar, but less marked, trend. Subfractions A probably consist mainly of myelin fragments and small nuclei (in the nuclear

subfraction), whilst subfractions D are probably composed of cell debris and larger nuclei. These fractions have not been investigated further.

The similarity in composition between the corresponding subfractions from the two parent fractions suggested cross-contamination of the fractions was occurring through inadequate washing. This conclusion was confirmed by washing the crude nuclear fraction by resuspension in fresh sucrose solution and centrifuging again for 15 x 10^3 g min. two or three times before carrying out the density gradient fractionation. Three such washes removed most of subfractions A, B, and C (Table 4).

Table 4. Protein content of subfractions prepared by density gradient centrifuging of washed nuclear fraction

Subfraction not sedimenting at 7.5 x 10^6 g min. through sucrose molarity stated		Protein mg./gm. tissue		
		Nuclear fraction washed 1 x	Nuclear fraction washed 2 x	Nuclear fraction washed 3 x
A,	1.0	0.90	0.27	0
B,	1.2	1.62	0.78	0.28
C,	1.4	2.22	0.80	0.28
D,	1.4	2.50	2.24	2.03

Morphological observations. Electron microscopy of the subfractions B from the nuclear and mitochondrial parent-fractions was very kindly undertaken by Dr. Williams of the Department of Anatomy, Guy's Hospital Medical School. The subfractions were fixed with osmium tetroxide and dehydrated by the method of Gray & Whittaker (1962), a procedure especially suitable for the demonstration of nerve endings in subcellular fractions. In confirmation of Gray & Whittaker's work, subfraction B of the mitochondrial fraction consisted mainly of nerve endings with some mitochondrial contamination. The nuclear subfraction B contained a few nerve endings and mitochondria, some amorphous material probably derived from disintegrated nuclei, and membrane-like structures of unknown origin. Thus electron microscopy gives added support to the conclusion that the nuclear subfraction B is similar to the corresponding fraction from the mitochondrial fraction, although it appears rather more heterogeneous.

Location of phosphoproteins sensitive to electrical stimulation

Introductory comment. At this stage of the work it appeared possible that the phosphoprotein sensitive to electrical stimulation might be associated with the 'pinched off' nerve endings described in dispersions of cerebral tissue by Gray & Whittaker (1962), for in a similar density gradient system to that used in our experiments these structures were found to equilibrate over 1.2 M-sucrose. On the other hand, since the fraction equilibrating over 1.2 M-sucrose is normally quantitatively some 5 times higher in the mitochondrial fraction than in the nuclear fraction, such a hypothesis might, at first sight, seem to be incompatible with Heald's earlier conclusion (Heald, 1959, made on the basis of direct observation of phosphoprotein radioactivity in stimulated slices) that the sensitive phosphoprotein was definitely not located in the mitochondrial or microsomal fractions. However, Heald's observations were of necessity made on dispersions of tissue which had been incubated in saline media in vitro and this fact, together with the high centrifugal force used to isolate the nuclear fraction, led to the unusual situation where some 50% of the total nitrogen and phosphoprotein of the dispersion, representing approximately 80% of the particulate matter, was recovered in the nuclear fraction (see also Heald, 1961). It is, therefore, difficult, on the basis of these results, to come to any firm conclusion regarding a location in any specific structure within the total particulate fraction.

Because of the limitations in carrying out satisfactory subcellular fractionation on incubated tissue by differential centrifuging, we initially decided to use a density gradient procedure which yielded two particulate fractions, one not sedimenting through 1.2 M-sucrose and approximately equivalent to subfraction B of the earlier study, and the other equivalent to the mitochondria-rich subfraction C. Whilst these experiments were in progress it was discovered that a more normal subcellular distribution of particulate matter from dispersions of incubated tissue could be obtained if adhering medium was first washed off the slices by rinsing them in a few ml. of sucrose solution before homogenization. This encouraged us to make further observations by the conventional procedures of differential centrifuging.

Comment on general procedure. In all these experiments the general procedure was as follows. Slices of guinea pig cerebral cortex were stimulated electrically for 10 sec. (alternating condenser pulses supplied at a 100/sec., peak voltage 15V, duration 0.4 msec. - see Ayres & McIlwain, 1953) in quick-transfer holders under the

conditions of Heald (1957). The incubation medium contained 3 μC of ^{32}P/ml. Immediately following stimulation the slices were rapidly transferred to ice-cold 0.32 M-sucrose and dispersed in an homogenizer. After fractionation in the ultracentrifuge, the subcellular fractions were treated with trichloroacetic acid and the specific radioactivity of the protein phosphorylserine determined. To obtain sufficient material for a single determination, it was necessary to pool several slices and precautions were taken to ensure that slices from different depths of cortex and from different animals were representatively pooled. Control slices were treated identically to stimulated slices, except that the stimulator was not switched on.

Stability of protein ^{32}P. It was first necessary to determine the stability in sucrose solution at 0° of ^{32}P incorporated into protein. After incubation and stimulation under the usual conditions, slices were rapidly dispersed in either 10% (w/v) trichloroacetic acid or cold 0.32 M-sucrose. The tissue in sucrose solution was then allowed to remain at 0° for various intervals before fixation with trichloroacetic acid. The results of two representative experiments are given in Table 5. Within 2 min. of dispersion in sucrose solution, the

Table 5. Loss of phosphoprotein-^{32}P in dispersions made in sucrose solution at 0°

Time after stimulation (min.)		Specific radioactivity of phospho-protein-P, counts/min./μg.P		Mean diff. in percentage stimulation
		Expt. 1	Expt. 2	
0	Control	32.6	30.0	55
	Stimulated	47.0	48.7	
2	Control	22.7	24.9	49
	Stimulated	32.5	37.4	
30	Control	23.9	21.2	41
	Stimulated	32.5	31.1	

specific radioactivity of the protein phosphorylserine in both control and stimulated slices fell to 70% of the value at zero time. Subsequently the phosphoprotein radioactivity changed relatively slowly. The difference in percentage stimulation between control and stimulated slices decreased by about 14 in 30 min. In an attempt to minimise the changes occurring in the first 2 min. a variety of

agents were incorporated in the sucrose solution. These included EDTA (at 5 mM concentration), ouabain (10^{-4} M), iodoacetate (10^{-3} M), p-chloromercuric benzoate (10^{-4} M), cyanide (10^{-3} M), mercuric chloride (10^{-4} M), ferric chloride (10^{-4} M); none of these agents prevented the loss in phosphoprotein-^{32}P, and some of them accelerated it. However, with ordinary 0.32 M-sucrose, the effect of electrical stimulation was still clearly evident after a period adequate to carry out at least a partial subcellular fractionation.

Observations on fractions isolated by density gradient procedures. Immediately after homogenization in 0.32 M-sucrose, the dispersion was centrifuged for 7.5 x 10^6 g min. over a discontinuous density gradient consisting of 7 ml. of 1, 8 ml. of 1.2, and 6 ml. of 1.4 M-sucrose, using the S.W.29 head of the Spinco Model L. Ultracentrifuge. The fractions equilibrating over the 1.2 M layer (B) and 1.4 M layer (C) were isolated for determination of protein, SDH, and phosphoprotein radioactivity. The other particulate fractions were discarded, as was the soluble protein remaining in the top layer of the 0.32 M-sucrose.

The mean results of three experiments are given below in Table 6.

Table 6. Effect of electrical stimulation on radioactivity of phosphoprotein in subcellular fractions isolated by a density gradient procedure

Fraction not sedimenting at 7.5 x 10^6 g min. through sucrose molarity stated		Protein mg./g. tissue	SDH μl CO_2/mg. protein/hr.	Specific radioactivity of phosphoprotein-P: counts/min./μg P	% increase in phosphoprotein radioactivity on stimulation
B, 1.2	Control	40.5	78	12.2	73
	Stimulated	39.5	70	21.2	
C, 1.4	Control	27.3	140	9.0	48
	Stimulated	26.0	145	13.3	

Mean of three experiments

The distribution of protein and SDH is very similar between fractions B and C in these experiments and mitochondrial subfractions B and C in the experiments using fresh tissue (Tables 2 and 3). Thus, in both cases there is some 50% more protein and one-half as much SDH in B than in C. The percentage increase in stimulation is clearly greater in all three experiments in fraction B than in C. A considerable response to stimulation is also seen in fraction C,

but in view of the inevitable cross-contamination occurring in the procedure, this was only to be expected. The results can at least be taken to exclude directly the possibility that a major increase in phosphoprotein radioactivity occurs in the mitochondrial proteins.

Observations on fractions isolated by differential centrifugation. Chemical analysis and electron microscopy of the subfractions from fresh tissue suggested that besides nerve endings and some mitochondria, subfraction B contained membrane material of microsomal origin. Further, it was evident that in the density gradient study, where unrinsed incubated tissue was used, microsomal material would also have occurred in this fraction, in all probability to an even greater extent. It therefore seemed important to attempt to isolate a microsomal fraction free of mitochondria or nerve endings. As was indicated above, this was only achieved when the importance was realised of rinsing the slices before dispersion. If slices were not rinsed, the yield of microsomal protein ranged in several experiments from 0 - 2 mg./g. of tissue; with rinsed slices, a yield of 10 - 14 mg./g. was regularly obtained, a value some 75% of that obtainable using fresh tissue.

Two particulate fractions were obtained by centrifuging dispersions of slices in 0.32 M-sucrose for 15 min. at 13,300 g, and then centrifuging the supernatant for 104,000 g for 60 min. In all but one experiment the original dispersion was also analysed. The approximate interval between stimulation and fixation with trichloroacetic acid was 15 min. in the cases of the original dispersion and the nuclear-mitochondrial fraction, and 75 min. for the microsomal fraction. Phosphoprotein radioactivity was determined as phosphorylserine as before, and the mean results from seven experiments are given in Table 7. No significant difference between control and

Table 7. Effect of electrical stimulation on phosphoprotein radioactivity in subcellular fractions obtained by differential centrifuging

Fraction	Protein mg./gm. tissue	SDH μl CO_2/mg. protein/hr.	Phosphoprotein-^{32}P counts/min./μg. P	% increase in phosphoprotein-^{32}P on stimulation
Original dispersion	86.8	108	Control 17.8 ± 1.9 Stimulated 23.1 ± 2.0	30 (± 8.6) ± 3.5
F1, nuclear & mitochondrial (2×10^5 g min.)	47	146	Control 15.3 ± 2.3 Stimulated 16.1 ± 2.9	5.2 (± 8.4) ± 3.2
F2, microsomal (6.2×10^6 g min.)	11.2	38	Control 17.6 ± 2.8 Stimulated 21.8 ± 2.9	23.7 (± 5.5) ± 2.3

Mean of seven experiments (six in the case of the original dispersion). Means are followed by their standard error.

stimulated slices was observed in the combined nuclear and mitochondrial fraction, whereas in the original dispersion and in the microsomal fraction there was a significant increase ($P < 0.01$) in phosphoprotein radioactivity on stimulation. We assume that the rather lower percentage stimulation found in the microsomal fraction compared with that found in the original dispersion was due to further losses of phosphoprotein-^{32}P occurring during the fractionation period. In one experiment not included in the series of Table 7, the stimulation was 29% in the dispersion, -1% in the nuclear-mitochondrial fraction, and 100% in the microsomal fraction. There is thus good evidence that the change in phosphoprotein radioactivity occurring on stimulation is a function of the microsomal phosphoprotein.

Studies of a cerebral protein phosphokinase

Introduction

Of the known systems in brain capable of phosphorylating proteins, that studied by Rabinowitz & Lipmann (1960) appeared to be of particular interest. This consisted of a partially purified protein phosphokinase, catalysing the transfer of ^{32}P from $AT^{32}P$ to phosvitin and casein and prepared from acetone powders of calf brain. Neither of these phosphoproteins is native to cerebral tissue and cannot be the natural substrate for the enzyme. However, it is very likely that proteins of a similar basic structure do occur in tissues (see Heald, 1961, and comment below on p. 17), and a study of the enzyme system using phosvitin as acceptor was felt to be justified.

Two aspects, not touched upon by Rabinowitz & Lipmann, have in the main been studied. These were first, determination of the subcellular distribution of the enzyme in cerebral tissues and, second, an investigation of the effect of cations on the partially purified enzyme. Cation effects were chosen in view of the possibility that the kinase enzyme, coupled with a protein phosphatase, could conceivably serve as part of a system hydrolysing ATP. As has been mentioned already, a sodium-stimulated ATPase is widely held to play an important role in cation transport in cerebral and other tissues.

General procedures. For assaying the enzyme, the basic medium contained 2 mg. of phosvitin, 50 μmoles of tris-HCl (pH 7.4), 1 μmole of $AT^{32}P$ (1×10^4 c.p.m./μmole of acid labile-P), and 1 - 5 μmoles of $MgCl_2$. In some early experiments, tris-phosphate buffer was used. The final volume, after addition of other cations and enzymes, was 1 ml. The incubation period at 37° was usually 10

min. The reaction was stopped with 4 ml. of 10% (w/v) trichloroacetic acid. The precipitated protein was then washed in the centrifuge 3 times with fresh trichloroacetic acid, dissolved in 1 ml. of N-NaOH, diluted to 10 ml. and counts due to ^{32}P determined in a liquid counter to an accuracy of ± 10%. Control samples in which addition of enzyme was omitted, normally gave counts less than 10% of the test samples.

The procedure used for preparing the soluble enzyme system from acetone-butanol powders of ox brain grey matter was essentially the same as that of Rabinowitz & Lipmann (1960). The fraction precipitated with 30% saturation with $(NH_4)_2SO_4$ was discarded and that precipitated by 50% saturation collected, dissolved in 0.01 M-tris HCl, pH 7, and dialysed against the same buffer at 0° until free of NH_4^+ ions. Dialysis did not affect enzyme activity.

Subcellular distribution of protein phosphokinase. When increasing amounts of a dispersion of guinea pig cerebral cortex in 0.32 M-sucrose were assayed for protein phosphokinase activity, the incorporation of ^{32}P into phosvitin increased linearly up to 500 μg. of protein and then fell off sharply. In assaying activity in subcellular fractions, therefore, the amount of protein added was limited to 200 μg. Subcellular fractions were prepared by differential centrifugation; nuclear and mitochondrial fractions were each washed once and the washings combined with the appropriate supernatant. Two experiments are reported in Table 8. The highest activity, both in absolute amount and in terms of unit quantity of protein, was found in the soluble fraction. Considerable activity was also present in the microsomal fraction.

Table 8. Distribution of protein phosphokinase in subcellular fractions of guinea pig cerebral cortex

Fraction	Total c.p.m. x 10^{-3} for whole fraction	c.p.m./μg. protein added	% recovered
Original dispersion			
Expt. 1	345	1.89	
Expt. 2	460	1.58	
Nuclear (8 x 10^3 g min.)			
Expt. 1	17	1.74	6
Expt. 2	33	1.58	7
Mitochondrial (2 x 10^5 g min.)			
Expt. 1	94	1.07	29
Expt. 2	112	0.82	24
Microsomal (9 x 10^6 g min.)			
Expt. 1	73	2.36	23
Expt. 2	93	1.66	19
Cytoplasm (soluble)			
Expt. 1	132	3.69	42
Expt. 2	254	4.50	50

Recovery: Expt. 1, 92%; Expt. 2, 107%. Medium: 1 mM-ATP, 5 mM-Mg, 200 mM-Na, 30 mM-K, 20 mM-tris-PO_4

The results in Table 8, expressed as counts/min., are equivalent to rates of 0.05 - 0.1 μmoles of P transferred/mg. of protein/hr. The reaction under these conditions is thus seen to be many times slower than the ATPase systems in brain (Schwartz, Bachelard & McIlwain, 1962).

Effect of cations on soluble enzyme system. The specific activity (μmoles of P transferred/mg. of protein/ hr.) of the soluble enzyme extracted from ox brain acetone powders was some 10 times higher than that of fresh dispersions of tissue. Enzyme activity was linear in the range 50 - 400 μg. of protein, but observations at higher levels were not made. For each enzyme preparation, the quantity of protein added was adjusted so as to give an incorporation in the test samples at least 5 times the counts found in the control sample; with most preparations, around 200 μg. was added. With an incubation time of 10 min., 1 - 5% of the γ-^{32}P of ATP was incorporated into the phosvitin.

The effect of Mg and Ca on enzyme activity is shown in Table 9. Maximum stimulation by the former is given at 5 mM-concentration, whereas Ca either has no effect or is inhibitory.

Table 9. Effect of Mg and Ca on protein phosphokinase

Enzyme prep. (protein added)	Conc. of Mg or Ca (mM)	μmoles of P/mg. protein/ hr.
2F (152 μg.)	Mg, 0; Ca, 0	0
	Mg, 5	0.47
	Ca, 3	0
	Ca, 7	0
	Mg, 5; Ca, 5	0
4A (230 μg.)	Mg, 1	0.06
	Mg, 2	0.14
	Mg, 3	0.27
	Mg, 5	0.39
	Mg, 7	0.35
	Mg, 8	0.26

Basic medium (1 ml.) contained: phosvitin, 2 mg.; AT^{32}P (1 x 10^4 c.p.m./ μmole acid labile-P), 1 μmole; tris-HCl, pH 7.4, 50 μmoles. Incubation time: 10 min.

The next Table (10) gives the percentage stimulation given by a variety of cations over and above that given by Mg alone. These results are representative of a number of experiments using different

enzyme preparations. Results from one preparation were obtained in the same experiment.

Table 10. Effect of various cations on protein phosphokinase

Enzyme preparation	(a) Mg-conc. (mM)	(b) Cation (200 mM)	% stimulation ($\frac{b}{a}$ x 100)
4B	5	K	160
	3	NH_4	300
	1	Li	101
	3	Li	120
	5	Li	89
4C	1	Na	740
	3	Na	435
	5	Na	160
	1	K	760
	3	K	490
	5	K	180
5B	3	Rb	390
	3	Cs	332
	3	Choline	180
9	3	Tris	190

Conditions were the same as in Table 9.

All of the monovalent cations tested, with the exception of Li, stimulated activity. The extent of the stimulation was markedly dependent on the concentration of Mg in the medium, decreasing as the total Mg was increased to 5 mM. This is shown in the Table for Na and K but exactly the same pattern was found for the other ions. Nevertheless, in the absence of added Mg, neither Na or K (at 200 mM) induced activity. The effect of varying concentrations of Na and K in the presence of 5 mM-Mg is given in Table 11.

Table 11. Effect of Na and K on protein phosphokinase

Na-conc. (mM)	µmoles P/mg. protein/hr.	K-conc. (mM)	µmoles P/mg. protein/hr.
40	0.44	40	0.49
100	-	100	0.68
200	0.76	200	0.91
300	0.73	300	0.81

Conditions were the same as in Table 9. Enzyme 5B.

Effect of phosphate ions A stimulation of the enzyme by phosphate ions (maximal at 60 mM) was reported by Rabinowitz & Lipmann (1960). No details of the corresponding cation used with the phosphate was given in their paper. In an early stage of the present investigation we found that addition of 60 mM-tris as the phosphate instead of 50 mM-tris-HCl had no effect on the enzyme, but stimulation occurred when the tris (as phosphate) concentration was increased to 100 - 200 mM (30 - 70 mM-phosphate ions). At the time this was taken to indicate a stimulation by phosphate, as at that stage we were unaware that tris ions stimulate the enzyme. Later it was shown that the stimulation given by 100 - 200 mM-tris (as phosphate) was closely paralleled by the effect of equimolar amounts of tris ion added as the hydrochloride. It would appear likely, therefore, that the stimulation of the enzyme by phosphate reported by Rabinowitz & Lipmann was due to the counter-cation used by them, although the latter was not specified. Lithium is the only cation we have found that does not stimulate, but lithium phosphate is very insoluble.

Effect of Na and K on protein phosphokinase enzymes in microsomes and cytoplasmic (soluble) proteins. For the above studies, enzymes from acetone powders, prepared from unfractionated cerebral cortex, had been used. In view of the wide subcellular distribution of the enzyme, it seemed important to determine whether the effect of cations was confined to the enzyme in a particular cell structure. Attention was, therefore, focussed on preparations made from microsomes and the soluble cytoplasmic proteins. The nuclear and mitochondrial fractions were not studied, as they are relatively more heterogeneous in composition. Both in fresh subcellular fractions and in soluble enzyme preparations made from acetone powders of the fractions, the effect of Na and K was similar to that found in the whole tissue preparations. Potassium was slightly more effective than Na in both cases, but the effect of either ion on the microsomal system was less reproducible. Some results obtained from fresh subcellular fractions are given below in Table 12.

Table 12. Effect of Na and K on enzyme in microsomes and cytoplasmic proteins

Mg-conc. (mM)	Stimulation (%) over control (Mg only) given by cation at 200 mM			
	Microsomes		Soluble proteins	
	Na	K	Na	K
2	255	340	340	370
3	213	262	241	307
4	153	229	177	177

Basic medium contained: phosvitin, 2 mg; $AT^{32}P$ (5×10^4 c.p.m./μmole of acid labile-P); tris-HCl pH 7.4, 50 μmoles. Incubation time, 10 min. 1 μmole

Guanosine triphosphate as precursor

Some evidence was obtained by Heald (1957) for an increase in the specific activity of GTP in cortical slices following electrical stimulation. A further study (Heald & Stancer, 1962) gave support to these findings and was held to support the hypothesis that GTP is a more immediate precursor of the tissue phosphoprotein-P than is ATP. Experiments were, therefore, carried out to determine whether $GT^{32}P$ is a more effective donor than $AT^{32}P$ in the phosvitin system. No difference between the two nucleotides could be detected. A representative experiment is shown in Table 13.

Table 13. Comparison of $AT^{32}P$ and $GT^{32}P$ as precursors for the protein phosphokinase system

Cations medium (mM)	μmoles P/mg. protein/hr.	
	$AT^{32}P$	$GT^{32}P$
Mg, 3	0.22	0.21
Mg, 3; Na, 200	0.64	0.69
Mg, 3; K, 200	0.78	0.71

Basic medium as in Table 9, except activity of $AT^{32}P$ was 0.9×10^4 c.p.m. and that of $GT^{32}P$ 1.2×10^4 c.p.m./μmole of acid labile-P. Conc. of both nucleotides was 1 mM.

Attempts to prepare protein fractions from brain enriched in phosphoproteins

Introduction

There has been little study as yet of the nature of the phosphoprotein fraction in mammalian tissues. Certain enzymes, for example phosphoglucomutase (Milstein & Sanger, 1961), alkaline phosphatase (Engstrom, 1961), and phosphorylase (Krebs, Kent & Fischer, 1958) have been isolated and found to contain one to four phosphorylserine residues. However, it is unlikely that proteins of this type account for all of the alkali labile phosphorous in the phorphoprotein fraction, which in brain equals approximately 10 μmoles of P/mg. of protein. Thus assuming that the tissue phosphoproteins are of molecular weight around 100,000 and contain

on an average 2 phosphorylserine residues per molecule, at least half of the tissue protein would have to be classed as phosphoprotein. It is more likely that the non-phosphorylated proteins constitute considerably more than half the total and the phosphoproteins consist of a mixture of proteins containing from one to many phosphorous atoms per molecule. This suggests the existence in tissues of phosphoproteins of the type represented by phosvitin or casein, which lack enzymic function and contain many P-atoms per molecule; although so far they have not been isolated.

A notable attempt by Heald (1961) was partially successful, but the product was probably denatured, at least partly, by the detergent used to solubilize the protein.

The problem presented by the electrical stimulation experiments was rendered more complicated by our ignorance, at the beginning of the Contract, of the precise subcellular site of the sensitive phosphoprotein, although it was clearly associated with particulate material. Therefore until its site was more precisely determined, it seemed advisable to confine our studies in this field to exploratory experiments. These have consisted mainly of an investigation into certain methods for bringing insoluble phosphoproteins into solution.

Preparation of insoluble protein

Instead of using a total particulate fraction for these studies, we chose the water-insoluble protein fraction remaining after exhaustive extraction of ox brain grey matter with 2.5 M-NaCl (see Le Baron & Folch, 1959). This fraction probably consists almost entirely of lipoproteins derived from the membranes of the nuclei, mitochondria, endoplasmic reticulum, and cell-walls. Thus when subcellular fractions of guinea pig cerebral cortex were extracted with 2.5 M-NaCl the percentage of protein extracted was 29 for the nuclear, 10 for the mitochondrial, [illegible] and 0 - 0.5 for the microsomal fraction.

The fraction was prepared as follows: ox brain grey matter was homogenized in ice-cold 2.5 M-NaCl (1 g. to 5 ml.) and the homogenate centrifuged for 4.7×10^6 g min. The insoluble lipoproteins were then washed twice by rehomogenizing in water and centrifuging again for the same time.

Solubilization procedures

Two approaches have been used: (a) ultra sound, and (b) alkaline pH. For the present we have avoided the use of external agents such as detergents, as the interactions of these substances with proteins and lipoproteins is relatively little understood.

(a) Ultra-sound. Using the MSE Ultrasonic disintegrator with a titanium probe (1.3 mA, 20 ks/sec.), 10 min. bursts with adequate cooling precautions, were given to water homogenates of the insoluble protein. The sonicated material was centrifuged for 7×10^6 g min. and the insoluble and soluble fractions analysed for protein, phosphoprotein (as alkali labile-P), and phospholipid-P. The following results were obtained.

Table 14. Effect of ultra-sound on ox brain insoluble protein

Fraction	Protein ~~solubility~~ %	Phosphoprotein-P mμmoles P/mg. prot.	Phospholipid-P mμmoles P/mg. prot.
Homogenate after sonication	100	4.2	1000
Soluble	14	5.2	452
Insoluble	86	3.3	904

Of the total protein, 14% was solubilized by this procedure and the soluble fraction was a little richer in phosphoprotein than the insoluble fraction. Compared with the soluble fraction produced by the next procedure, the sonicated soluble material was relatively higher in phospholipids. The soluble fraction obtained by this procedure tended to become less soluble on standing at 0° for 24 hr. or longer.

(b) Alkaline pH. This procedure was suggested by observations on erythrocyte membranes by Moscowitz & Calvin (1952), and Klouet & Richter (1959) on brain microsomes. In preliminary experiments, the insoluble proteins were homogenized at 0° in a variety of agents, the final pH in all cases falling between 10 and 10.5. A soluble fraction was obtained by centrifuging as before; the insoluble fraction was discarded. Results are given in Table 15.

By far the most effective agent was NaOH, and provided that the temperature was kept at near zero throughout the operation, very little protein dephosphorylation occurred. The soluble product is notably low in phospholipid-P.

The use of 0.01 M-NaOH was, therefore, explored further. It was found feasible to bring the pH of the homogenate back to 7.4 with 0.1 N-acetic acid before centrifuging, without any

reprecipitation of the solubilized protein. When the pH of the soluble fraction was reduced to 4.5 with acetic acid, some 86% of

Table 15. Effect of various agents at pH 10 on protein solubility

Agent (100 vol.)	Protein solubility	Phosphoprotein-P mμmoles P/mg. prot.		Phospholipid-P mμmoles P/mg. prot.	
		Total	Soluble	Total	Soluble
NaOH 0.01 M	26	6.52	5.78	875	41
NH_4OH 0.03 M	15	-	6.47	973	46
Amm.acetate 0.1 M	12	7.5	5.12	875	41
Tris 0.1 M	8	6.02	4.13	840	27
NaOH-glycine buffer 0.1 M	5	6.34	5.93	788	107

the protein was precipitated. Quite a high proportion of this precipitated protein was apparently denatured, as it could not be brought back into solution even when the pH was raised to 9; the soluble fraction remaining, however, was slightly enriched in phosphoprotein. The general procedure was as follows.

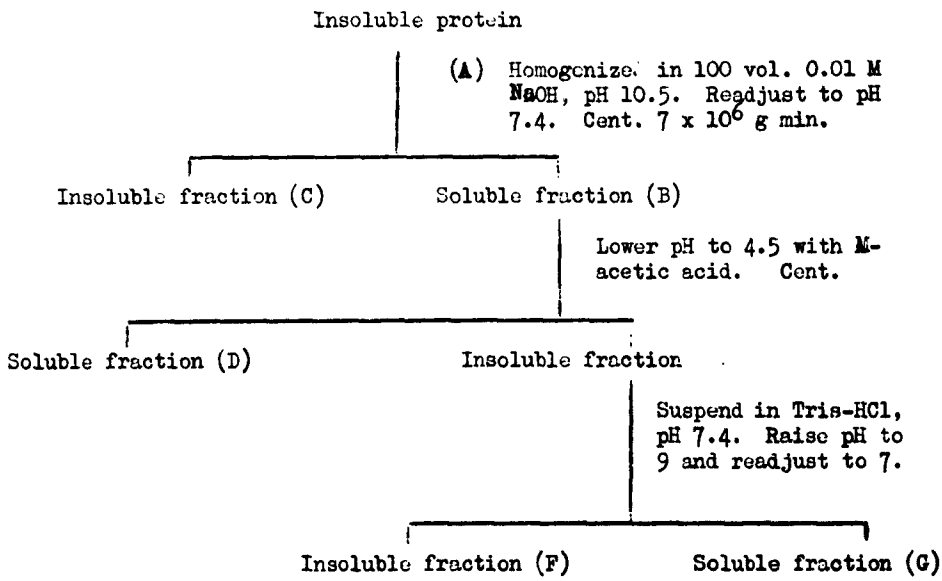

Table 16 gives the analyses of fractions A - G from this scheme.

Table 16. Analysis of fractions in solubilization procedure

Fraction	Protein solubility %	Phosphoprotein-P mμmoles P/mg.prot.	Phospholipid-P mμmoles P/mg.prot.
A	100	7.9	763
B	25 of A	7.4	178
C		7.5	1320
D	14 of B	3.4	6
F		6.5	258
G	18 of B	8.3	22

Application to microsomes

The experience gained with the insoluble protein fraction from ox brain has been applied in preliminary experiments to guinea pig microsomes.

The first stage of the procedure was carried out at 0° with continuous measurement of pH. NaOH (0.1 M) was added dropwise to a vigorously stirred suspension of microsomes in 0.32 M-sucrose until the pH reached 10.5. The pH was then immediately brought down to 7 with 0.1 M-acetic acid and the suspension centrifuged at 2.3×10^6 g min. The soluble fraction was analysed at this stage, the further precipitation at pH 4.5 being omitted for these preliminary experiments. Results of such an experiment are given in Table 17.

Table 17. Solubilization of microsomal protein by treatment at pH 10.5

Fraction	Protein (mg.)	Phosphoprotein-P mμmoles P/mg. prot.
Microsomes	140	14.2
Soluble	16	18.6
Insoluble	124	12.6

The solubility attained by this method (11%) is less than was found with the ox brain proteins, but some enrichment in phosphoprotein in the soluble fraction is seen. When the

insoluble protein was treated for a second time at pH 10.5, a further 7% of the protein was solubilized and again the soluble fraction contained more phosphoprotein, the figures for the soluble and final insoluble residue being 15.9 and 10.8 mμmoles P/mg. protein respectively. Attempts to increase the yield of soluble protein by raising and lowering the pH three times without intervening centrifugation were unsuccessful.

In a further experiment, the microsomal phosphoproteins were labelled by incubation of the suspension for 5 min. with $AT^{32}P$, $MgCl_2$, and tris buffer pH 7.4 before solubilization. After centrifuging and resuspending the microsomes in 0.32 M-sucrose, a soluble fraction was obtained as above. From Table 18 it can be seen that the phosphoproteins in the soluble fraction are labelled at least as much as in the insoluble fraction.

Table 18. Labelling of microsomal proteins with $AT^{32}P$

Fraction	Protein (mg.)	Phosphoprotein-^{32}P c.p.m./μg P
Microsomes	17	765
Soluble	1.7	1002
Insoluble	15	766

It should be pointed out, however, that the phosphoprotein radioactivity in these experiments was determined from alkali labile-P and not from phosphorylserine analysis.

Discussion

Subcellular location of phosphoproteins

The present results strongly suggest that at least part of the phosphoprotein response to electrical stimulation, observed in whole tissue homogenates, occurs in a microsomal phosphoprotein. The earlier observations of Heald (1959) that pointed to a location in structures sedimented with the nuclear fraction were apparently due to the inclusion of microsomal material in the nuclear fraction, which occurs as a result of contamination of the sucrose dispersing-solution with traces of incubation medium carried over with the slice.

A point requiring discussion is the relatively lower percentage increase in phosphoprotein radioactivity occurring on stimulation found in the microsomal fraction compared with the value found in dispersions

of the whole tissue (Table 7), for if the microsomal proteins were the only fraction to change, one would expect a greater difference in this fraction than in the total cellular phosphoprotein. It is possible, however, that the induced increase in radioactivity of the microsoma. phosphoproteins declines more rapidly during the subcellular fractionation than is suggested by the data of Table 5, which was obtained by analysis of whole tissue. Unfortunately there is no way at present of testing this hypothesis: a possible approach would be to search for agents to inhibit the supposed loss of newly-incorporated ^{32}P without affecting the subcellular fractionation.

There is another reason why it is not absolutely certain that the microsomal phosphoprotein is the only fraction to change on stimulation. This is that the mitochondrial fraction in brain is the principle site of a phosphoprotein phosphatase, whereas very little of this enzyme is found in microsomes (Rose, 1962). Thus the possibility has not been excluded that a mitochondrial phosphoprotein sensitive to stimulation becomes dephosphorylated by this enzyme during the isolation of the fraction. However, very little is known as yet about the metabolism of phosphoproteins, and it is quite possible that they are involved in both energy-yielding and energy-utilising processes. Thus the present findings indicate a role for these molecules in energy utilization, but do not finally exclude participation in the energy-yielding recovery processes in the cell following excitation.

Whether the microsomal phosphoprotein response is directly related to ion transport or to other events in the membrane remains to be elucidated. Strong support for the former interpretation comes from a recent study by Ahmed, Judah & Wallgren (1963), who found that either ouabain, a known inhibitor of ion transport across cell membranes, or the substitution of Li for Na in the medium, both block the effect of electrical stimulation on phosphoprotein radioactivity.

Cerebral protein phosphokinase enzyme

Subcellular distribution. The protein phosphokinase phosphorylating phosvitin is widely distributed in the cell in a somewhat similar pattern to that of the tissue phosphoprotein fraction. Possibly the enzyme has a general function in phosphorylating phosphoproteins of a certain type, wherever they occur. However, it must be emphasised that there is no firm evidence (a) that enzyme activity in different subcellular fractions is due to the same enzyme, or (b) that proteins of the type represented by phosvitin occur in the cell, although, as indicated on page 17, this is a distinct possibility. So far as stimulation by Na and K is concerned, the enzyme in microsomes and cytoplasmic proteins cannot be distinguished.

The rate of phosphate transfer shown by the enzyme system with phosvitin as substrate is many times less than that of the several ATPase systems in the cell. Unless the kinase reacts with a natural substrate at a much higher rate, it is difficult to see how it could couple with a protein phosphatase to give an ATPase system of the required activity.

Effect of cations. These studies also tend to discount a role for the kinase enzyme in ATPase action. Thus the membrane ATPase in brain (Schwartz, Bachelard & McIlwain, 1962) is stimulated by sodium maximally in the presence of 30 mM-potassium, whilst K itself does not stimulate. The protein phosphokinase, on the other hand, is stimulated by K as well as many other cations including tris.

It would be unprofitable to speculate on the possible mechanisms by which cations stimulate the protein phosphokinase enzyme, since the present enzyme system is relatively impure. It is perhaps worth while pointing out that the cations may possibly be modifying the physico-chemical properties of the phosvitin substrate as well as interacting with ATP and the enzyme.

Attempts to prepare protein fractions enriched in phosphoproteins

The problems involved in these aspects of the work fall into two categories: (a) that of solubilizing the microsomal protein without degrading the phosphoproteins, and (b) that of fractionating the soluble material.

Regarding solubilization, we have avoided for the present the use of detergents, in view of the complex and only partly understood action of these substances on lipoproteins (Fox, 1957). It is possible, however, that certain of the membrane proteins may possess no true solubility in water except in the presence of molecules with surface active properties (see Green et al. 1961). Further, it must be recognised that the present procedure of solubilizing at pH 10.5 only brings into solution a relatively small proportion of the microsomal protein. So far as the phosphoprotein solubilized is concerned, we have as yet no evidence as to whether this fraction is representative of the total membrane phosphoprotein or whether it is a different fraction more readily solubilized.

In the case of fractionation procedures, some preliminary experiments conducted on soluble cytoplasmic proteins from brain suggest that chemical methods of fractionation such as neutral salts and organic solvents will be of limited value only. Chromatographic and electrophoretic techniques show more promise.

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